IDENTIFICATION OF CHEMICAL MARKERS AND QUALITY ASSESSMENT IN MALAYSIAN STINGLESS BEE HONEY USING CHEMOMETRIC TECHNIQUES

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

%	percentage
μg/mL	micrograms per millilitre
μL	microlitre
μm	micrometre
°C	degree Celsius
°C min^{-1}	degree Celsius per minute
cm^{-1}	per centimetre
g	gram
h	hour
M	molar
meq/kg	milliequivalents per kilogram
mg	milligram
mg/kg	milligrams per kilogram
mg/L	milligrams per litre
min	minute
mL	millilitre
mL min^{-1}	millilitres per minute
mm	millimetre
mM	millimolar
mS/cm	milliSiemens per centimetre
mV	millivolt
N	normality
nm	nanometre
RM	Ringgit Malaysia

rpm	revolutions per minute
w/v	weight per volume

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrometer
ACE	Angiotensin-converting enzyme
Al	Aluminium
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
As	Arsenic
Ba	Barium
Ca	Calcium
CE	Capillary electrophoresis
Cd	Cadmium
CO_2	Carbon dioxide
Cr	Chromium
Cu	Copper
DPPH	2,2-diphenyl-1-picrylhydrazyl
e.g.	For example
EU	European
Fe	Iron
FID	Flame ionisation detector
FRAP	Ferric reducing antioxidant power
GC-MS	Gas chromatography–mass spectrometry
Hg	Mercury
5-HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
ICP-AES	Inductively coupled plasma-atomic emission spectroscopy

ICP-MS	Inductively coupled plasma-mass spectroscopy
ICP-OES	Inductively coupled plasma-optical emission spectrometry
i.d.	Internal diameter
i.e.	That is
K	Potassium
LLE	Liquid-liquid extraction
Mg	Magnesium
Mn	Manganese
MIP-AES	Microwave plasma-atomic emission spectroscopy
Mo	Molybdenum
MS	Mass spectral
Na	Sodium
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Ni	Nickel
P	Phosphorus
Pb	Lead
PTFE	Polytetrafluoroethylene
Rb	Rubidium
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
Se	Selenium
Sr	Strontium
UV	Ultraviolet
V	Vanadium
Vis	Visible
Zn	Zinc

LIST OF APPENDICES

PENENTUAN PENANDA KIMIA DAN PENILAIAN KUALITI DALAM MADU LEBAH KELULUT MALAYSIA MENGGUNAKAN TEKNIK KEMOMETRIK

ABSTRAK

Madu *Heterotrigona itama* (*H. itama*) adalah madu kelulut yang dihasilkan oleh spesies lebah kelulut yang bernama *H. itama*. Bagi memasarkan madu *H. itama* secara komersial di pasaran tempatan dan antarabangsa, pematuhan terhadap parameter penilaian kualiti madu adalah penting. Dalam kajian ini, parameter penilaian kualiti yang dinilai adalah kandungan kelembapan, kandungan abu, kekonduksian elektrik, pH, keasidan bebas, jumlah pepejal terlarut, dan kandungan 5-hidroksimetilfurfural (5-HMF) dalam madu *H. itama* yang dikumpulkan dari tiga negeri yang berbeza (Pulau Pinang, Kedah, dan Perak) telah dinilai dalam keadaan segar dan selepas enam bulan pada dua suhu penyimpanan berbeza (4 dan 30 °C) dan nilai-nilai yang diperoleh dibandingkan dengan piawaian Malaysia serta antarabangsa. Sebagai tambahan kepada parameter penilaian kualiti, kandungan fenolik dan flavonoid keseluruhan, serta komposisi pelbagai elemen juga telah dikaji. Kemudian, satu kajian pembezaan geografi dijalankan dengan menggunakan data daripada analisis tersebut yang digabungkan dengan sebatian organik senang meruap dalam madu mengikut penggunaan analisis multivariat. Analisis penilaian kualiti semasa keadaan segar menunjukkan bahawa kandungan lembapan, kandungan abu, keasidan bebas, dan jumlah pepejal terlarut kebanyakan sampel gagal memenuhi keperluan yang ditetapkan oleh piawaian antarabangsa. Dari segi Piawaian Malaysia, kandungan abu semua sampel melebihi had. Walaupun terdapat elemen surih yang beracun (Cd, Ni, dan As) yang dikenalpasti dalam sebilangan sampel, pengambilan pelbagai elemen

secara harian/mingguan yang dianggarkan masih di bawah pengambilan harian/mingguan yang boleh diterima oleh tubuh manusia. Selepas enam bulan penyimpanan pada 30 °C, kekonduksian elektrik lebih banyak sampel melebihi piawaian antarabangsa dan pH kebanyakan sampel melebihi pH yang disyorkan oleh Piawaian Malaysia tetapi masih dalam piawaian antarabangsa. Selain itu, selepas penyimpanan pada 30 °C, kandungan 5-HMF dalam semua sampel meningkat tetapi masih dalam Piawaian Malaysia dan antarabangsa. Selepas enam bulan disimpan pada suhu 4 °C, pH kebanyakan sampel melebihi Piawaian Malaysia. Pada kedua-dua suhu penyimpanan, kandungan kesuluruhan fenolik sampel meningkat, manakala kandungan keseluruhan flavonoid, kandungan abu dan kandungan pelbagai elemen menurun. Pada masa yang sama, kajian ini juga mendedahkan bahawa kandungan keseluruhan fenolik berkorelasi positif dengan aktiviti penghapusan radikal bebas (r = 0.749) dan kuasa pengurangan (r = 0.739). Selain itu, analisis diskriminan bersepadu separa menunjukkan corak pembezaan sampel yang baik dan pengesahan persilangan 10 kali (R^2 = 0.92, Q^2 = 0.85, dan ketepatan = 0.97) dan ujian permutasi ($p < 0.05$) menunjukkan bahawa model tersebut sah. Selain itu, plot skor daripada kepentingan pembolehubah unjuran menemui bahawa empat belas sebatian organik boleh digunakan sebagai penanda kimia yang berkuasa (penunjuk untuk membezakan dua atau lebih keadaan) dan Fe, Mn, kandungan 5-HMF, dan kandungan keseluruhan flavonoid sebagai pembolehubah yang paling berpengaruh untuk geografi diskriminasi madu *H. itama*. Analisis diskriminan bersepadu separa orthogonal menunjukkan bahawa kandungan Cu dan 5-HMF boleh digunakan untuk membezakan sampel segar dan disimpan pada 4 °C. Di samping itu, 5-HMF, pH, kandungan keseluruhan fenolik, kekonduksian elektrik, Cu, dan Mg boleh digunakan untuk membezakan sampel segar dan disimpan pada 30 °C.

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ABSTRACT

Heterotrigona itama (*H. itama*) honey is stingless bee honey produced by stingless bee species of *H. itama*. For *H. itama* honey to be commercialised in local and international markets, the adherence of honey to the quality assessment parameters is vital. In this study, moisture content, ash content, electrical conductivity, pH, free acidity, total dissolved solids, and 5-hydroxymethylfurfural (5-HMF) content in *H. itama* honey collected from three different provinces (Pulau Pinang, Kedah, and Perak) were assessed during its fresh state and after six months at two different storage temperatures (4 and 30 °C) and the obtained values were compared with Malaysian and international standards. In addition to quality assessment parameters, total phenolic and flavonoid contents, and multi-element compositions also were investigated. Subsequently, a geographical discrimination study was carried out using data from the mentioned analysis incorporated with volatile organic compounds in honey using multivariate analysis. The quality assessment analysis during fresh condition revealed that moisture content, ash content, free acidity, and total dissolved solids of most samples failed to meet the requirement set by international standards. In terms of the Malaysian Standard, the ash content of all samples exceeded the limit. Besides, although toxic minor elements (Cd, Ni, and As) were present in some samples, the estimated daily/weekly intake of these multi-elements was still below tolerable daily/weekly intake. After six months of storage at 30 °C, the electrical conductivity of most samples exceeded the international standards and the pH of most

samples exceeded the recommended pH by Malaysian Standard but still within international standards. Moreover, 5-HMF content in all samples increased but still within both Malaysian and international standards after storage at 30 °C. After six months of storage at 4 °C, the pH of most samples exceeded Malaysian Standard. At both storage temperatures, the total phenolic content of samples increased, whereas the total flavonoid content, ash content and content of multi-elements declined. The study also demonstrated that total phenolic content positively correlates with free radical scavenging activity ($r = 0.749$) and reducing power ($r = 0.739$). Besides, the partial least squares-discriminant analysis showed a good discrimination pattern of samples and the 10-fold cross-validation ($R^2 = 0.92$, $Q^2 = 0.85$, and accuracy = 0.97) and permutation tests ($p < 0.05$) revealed that the model is valid. Furthermore, the variable importance of projection score plot discovered that fourteen volatile organic compounds could be used as potent chemical markers (indicators to distinguish two or more environments) and Fe, Mn, 5-HMF content, and total flavonoid content as the most influential variables for geographical discrimination of fresh *H. itama* honey. Orthogonal partial least square-discriminant analysis revealed that Cu and 5-HMF content could be used to distinguish fresh and stored samples at 4 °C. In addition, 5-HMF, pH, total phenolic content, electrical conductivity, Cu, and Mg could be used to distinguish fresh and stored samples at 30 °C.

CHAPTER 1

INTRODUCTION

1.1 Overview of chapter

The introduction chapter of this thesis serves as the gateway, providing a clear understanding of the research's context, significance, objectives, and scope. In the general background of the introduction chapter, bees and their common product (i.e., honey), compositions of honey, and therapeutic properties of honey were discussed. Consequently, the problems (research questions) that have arisen within the process of commercialisation and due to limited production with the availability of stingless bee honey have been mentioned clearly. In conjunction with the problems, a few main objectives that establish the rationale for conducting the study have been identified and listed. Lastly, the scope of the current research includes the analyses and type of storage conditions and shortcomings during the research work with their implications have been discussed.

1.2 General background

Figure 1.1: Artificial beehive at kelulut honeybee farm, Lenggong, Perak

Honey is an organic mellifluous substance that is usually exemplified by its complex composition (Abeshu & Geleta, 2016; Dao et al., 2019). The complex composition of honey is influenced by climatic conditions (temperature, humidity, precipitation, and sunlight), botanical origin, geographical origin, bee species, season, storage period, extraction technique, and handling method (Kadri et al., 2017; Sant'ana et al., 2020). Although the composition of honey varies, the sources of honey are limited to three. The sources are the nectar of flowers, the secretion of living components of plants, as well as excretion of plant-sucking bugs (Özcan et al., 2015). Without the aid and vital role played by herbivorous pollinator insects (also known as honeybees), honey is impossible to be produced naturally. After the collection of nectar, secretion, and excretion by honeybees, the naturally occurring enzymes of honeybees are responsible for transforming the substances, which are then deposited, dehydrated, and stored in the honeycomb till it ripens and matures to be extracted (Adenekan et al., 2010). The honeycombs are composed of numerous hexagonal prismatic wax cells and are typically made by honeybees (Yang et al., 2021). In the event of human-made bee apiaries, the beehives are usually artificially made by the beekeepers using wood (Figure 1.1) since it is easily available, eco-friendly, and provides a comfortable environment for the honeybees.

Honey is available in most countries around the world with different names, which vary according to the species of bees, blossom origin, and geographical origin. Well-known honey includes tupelo honey, manuka honey, acacia honey, leatherwood honey, honeydew honey, chestnut honey, clover honey, buckwheat honey, *Eucalyptus lanceolatus* honey, *Helianthus annuus* honey, and heather honey (Abbas et al., 2021; Nayik & Nanda, 2015; Yang et al., 2020). Although there are plenty of scientific reports comparing and contrasting the quality and standard of this honey, the demand for the types of honey among consumers is still based on visual colour intensity, aroma, viscosity, and the presence of remains (Ghorbani et al., 2009).

On the other hand, the global market for honey is vast and complex, with many factors manipulating the availability of honey at a particular time. Honey availability on a global scale is mainly determined by supply and demand. Honey production varies from year to year, depending on circumstances such as weather conditions, honeybee health, floral availability, and geographic location. For example, in the year 2019, the People's Republic of China led the international total honey production with 24% out of 0.002 billion tons (FAO Statistical Database, 2020). Besides China, other countries which successfully secured a place in the top 10 production list board (Figure 1.2) were the Argentine Republic, Canada, the Republic of India, the Islamic Republic of Iran, the United Mexican States, the Russian Federation, the Republic of Turkey, Ukraine, and the United States of America (FAO Statistical Database, 2020).

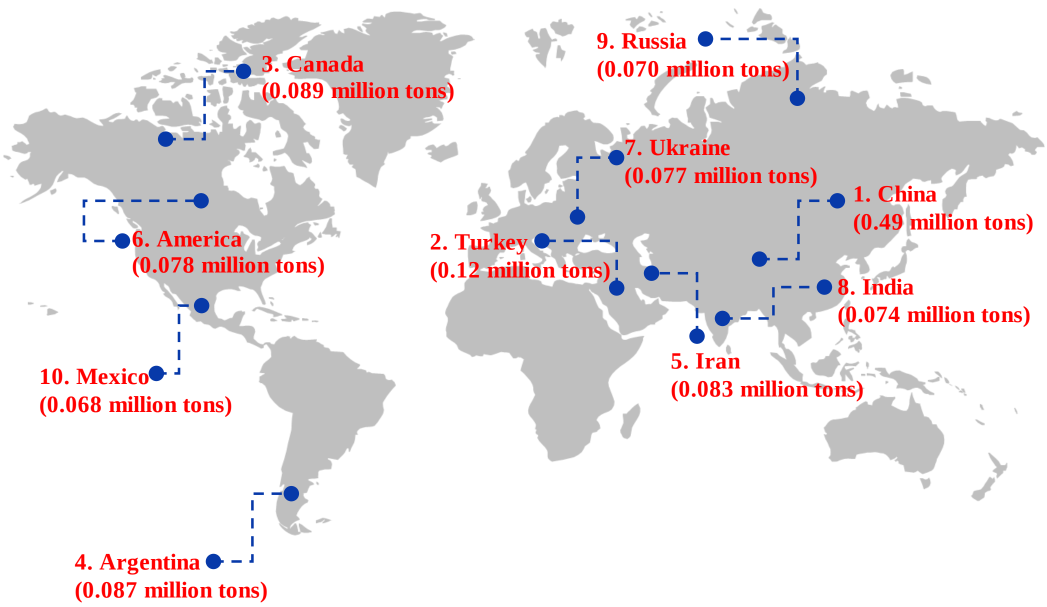

Figure 1.2: Top 10 countries with the highest production of honey in the year 2019

The major components of honey are carbohydrates and water, with a composition of 85 and 15%, respectively. Besides that, the minor constituents of honey are enzymes (glucose oxidase, invertase, and diastase), proteins, amino acids, minerals, organic acid, water-soluble vitamins, lipids, pollen grain, phytochemicals (carotenoids, phenolic acids, and flavonoids), waxes, and final products from the Maillard reaction (e.g., 5-hydroxymethylfurfural (5-HMF), 2-furaldeyhde, 3-furaldeyhde, 5-methylfuraldeyhde, 2-furoic acid, 3-furoic acid, and melanoidins)

(Duru & Duru, 2021; Gomes et al., 2010; Singla et al., 2018; Yong Foo et al., 2012). The natural sweetness of honey is due to the presence of carbohydrates as the major component (Linkon et al., 2015). The common individual components of the carbohydrates found in honey are monosaccharides (i.e., fructose and glucose) and disaccharides (i.e., maltose and sucrose) (Figure 1.3) (Wang et al., 2010). In addition, the rare monosaccharides found in honey are arabinose, glucose, fucose, rhamnose, ribose, galactose, mannose, xylose, galacturonic acid, and glucuronic acid (Lu et al., 2017). The percentage of each component of honey upon extraction varies according to the diet of bees, botanical and geographical origins, harvesting seasons, and extraction techniques (Alvarez-Suarez et al., 2014; Karabagias et al., 2014; Silva et al., 2013). Apart from being utilised as a sugar and flavour enhancer in food-producing industries, honey has also been used widely by consumers because of its nutritional and medicinal properties (Khan et al., 2007).

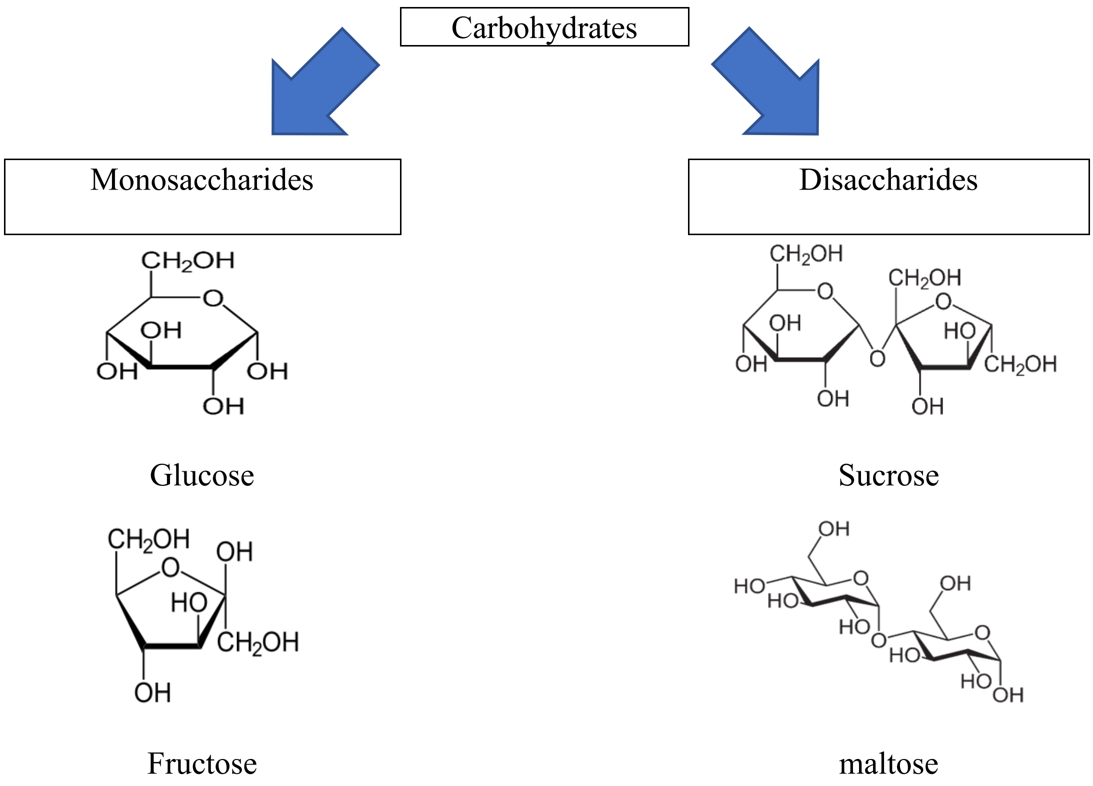

Figure 1.3: Common carbohydrates in honey

Globally, honey is categorised into two groups: monofloral honey and polyfloral honey (Vulić et al., 2015). The source of monofloral honey is the nectar obtained from the blossoms of a single plant by honeybees, while the source of polyfloral honey is the nectar obtained from blossoms of more than one single plant (Vulić et al., 2015). Polyfloral honey possesses a more complex flavour compared to unifloral honey, as it consists of a mixture of nectars from different plants (Aboud et al., 2011). Moreover, both farm and wild jungle honey can be consumed raw and unprocessed because of their non-allergic properties and due to human bodies being able to assimilate it easily; on top of that, the processed honey tends to have low nutritional properties (Blasa et al., 2006; El Sohaimy et al., 2015). The most commonly reported medicinal benefits of honey include treating wounds (such as burns, ulcers, and injuries), preventing osteoporosis, reducing the severity of asthma, curing cold and cough, and healing gastrointestinal problems (Eteraf-Oskouei & Najafi, 2013; Kumar & Bhowmik, 2010). In addition, honey is a multivitamin energiser that has anti-ageing, antifungal, anti-inflammatory, antioxidant, antiviral, and antimicrobial properties (Kumar et al., 2010).

Malaysia constitutes approximately 328,550 km^2 of land area, equivalent to 0.2% of the world's territory mass, notwithstanding the tropical rainforests, oceans, and freshwater environments that uphold different clusters of flora and fauna. The lush vegetation of the Malaysian rainforest is undoubtedly among the most extravagant on the planet and harbours numerous diverse varieties of nectar. There are a few common honey types in Malaysia, such as tualang honey, melaleuca honey, stingless bee honey, Borneo honey, pineapple honey, and acacia honey (Khalid et al., 2018; Moniruzzaman et al., 2013b; Zainol et al., 2013).

Generally, bees responsible for honey production can be partitioned into two general groups: sting bees and stingless bees (Figure 1.4). Unlike the other group, the sting bees, stingless bees (which originated from meliponini tribe) have evolved a unique way of defending themselves that does not involve a stinger (Shanahan & Spivak, 2021). These stingless bees are small in size, and it becomes their strength. This is because that uniqueness helps them to pollinate small-sized blooms (Vit et al., 2013). In addition, the difficulty of controlling stingless bees is much lower compared to other types of bees, which can easily get infected with diseases (Jalil et al., 2017).

Moreover, stingless bees do not harm people; thus, it is simpler to extricate the nectar from the beehive. Up to the present time, there are about 600 species of stingless bees and 61 genera found on the planet (Arung et al., 2021). These stingless bees are mostly distributed in areas that have a hot and humid climate, such as Southeast Asia (Malaysia, Thailand, and Indonesia), Australia, Africa (Ethiopia), South America (Federative Republic of Brazil and Venezuela), North America (United Mexican States) and Central America (Republic of El Salvador, Guatemala, Argentina, and Costa Rica) (Kidane et al., 2021; Shamsudin et al., 2019a; Silva et al., 2013; Sommeijer et al., 2003; Vit et al., 2013). The honey produced by stingless bees is known as stingless bee honey and the Malaysian stingless bee honey has become popular and is one of the most distributed honey locally after its accreditation and acknowledgement as a superfood by the Malaysian Agricultural Research and Development Institute (MARDI).

(a) (b)

Figure 1.4: (a) Stingless bee (Jaymi, 2021) and (b) Sting bee (Chawla, 2019)

1.3 Problem statement

The availability and supply of pure Malaysian stingless bee honey are limited due to several reasons. First and foremost, Malaysian stingless bee honey is an organic product and cannot be produced synthetically (Khan et al., 2018). Besides, the production of stingless bee honey is also lesser than other honey because of the small size and colonies of stingless bees (Lima et al., 2016; Roubik, 2023). The increasing demand for Malaysian stingless bee honey among consumers also became a limitation that led to the availability of honey. The high demand is because individuals are more health conscious about the nutritional and therapeutic characteristics of honey and how they may benefit from consuming it, especially to boost their immune system against SARS-CoV-2 during pandemics and diseases (Al-Hatamleh et al., 2020). Eventually, due to the limited supply and availability of stingless bee honey in the market, this causes an increase in the selling price of this honey (which can cost between RM 120 to RM 300 per kg); therefore, consumers approach beekeepers for direct purchasing stingless bee honey. Although directly obtaining honey from beekeepers by consumers can be a sustainable and cost-effective method, it can also pose a risk to the quality and safety of the product. This is because farmers may not have the necessary resources or knowledge to ensure the proper handling and processing of honey, such as extraction, straining, filtering, thermal treatment, etc. Besides, adulteration of honey is also a threat to consumers' health, but it is only vital when the source of honey is unknown and questionable. However, when the honey is obtained from authorised sources, the adulteration concern or aspect can be overlooked. Therefore, in order to commercialise stingless bee honey locally and internationally, the initial quality assessment of the honey and quality assessment values adherence to Malaysian and international standards are vital. Although two studies on adherence of *H. itama* honey to standards, the 5-HMF content of samples from these two studies shows a significant difference between them; thus, studies of 5-HMF content in more samples from Malaysia are required to illustrate whether samples from Malaysia will generally have higher or lower 5-HMF content (Ng et al., 2021, Omar et al., 2019). Moreover, both of these studies never include samples from the northern region of Peninsular Malaysia. It is important to note that both of these studies also failed to investigate the composition of multi-elements in *H. itama* and correlate them with the consumption safety of honey.

Besides, as a result of low production, supply, and price factors, Malaysian stingless bee honey is stored for a long period. For example, it is kept for a few months for daily consumption at home. During the storage period, the physical and chemical compositions of honey may change over time (Silva et al., 2016). The changes in physical and chemical compositions are mainly due to fermentation, volatilisation, oxidation, and thermal processing (Eshete et al., 2019; Silva et al., 2016; Xiang et al., 2019). Consequently, this may deteriorate the nutritional contents and quality safety to be used for medicinal and therapeutic benefits. On the other hand, the deterioration of volatile organic compounds could occur and this may cause the honey to have an off-flavour (Diez-Simon et al., 2019). Up to this moment, there is only one study has been reported on changes in the physicochemical properties of *H. itama* honey. However, the investigated storage period was up to 90 days (which is quite short) and ash content, electrical conductivity, and multi-element content were not even included in their storage study (Julika et al., 2022).

On the other hand, there are several studies on the physicochemical properties and chemical constituents of stingless bee honey vary according to entomological origins, botanic origins, and geographical origins (Costa et al., 2018; Shamsudin et al., 2019a; Sharin et al., 2021; Silva et al., 2017). However, to our best knowledge, there are no studies on identifying potent putative markers or the most influential variables based on physicochemical characteristics and chemical components (5-HMF, volatile organic compounds, and multi-elements) that can be used to distinguish *H. itama* honey from different geographical origins.

1.4 Research objectives

The first goal of this research is to identify the compliance of designated parameters with international and Malaysian standards during the fresh state and the changes during the different storage conditions. Secondly, to carry out supportive analyses to explore and support the correlation between total phenolic and flavonoid contents with antioxidant and anti-hypertensive properties of honey. Lastly, the subsequent goal is to identify the most influential variables and putative chemical markers.

i. To analyse Malaysian stingless bee honey's physicochemical characteristics, 5-HMF, total phenolic content, total flavonoid content, multi-elements, and volatile organic compounds under fresh and after storage conditions.

ii. To investigate the antioxidant properties and angiotensin-converting enzyme (ACE) inhibitory assay of selective samples.

iii. To apply chemometric techniques to the investigated parameters for geographical with storage discrimination study and putative chemical markers identification.

1.5 Scope and limitation of the study

Based on international and local standards, there are a total of nine parameters for honey quality assessment, such as moisture content, pH, free acidity, electrical conductivity, ash content, 5-HMF, diastase activity, sucrose, and the sum of glucose with fructose (Bogdanov et al., 1999; Department of Standards Malaysia, 2017). However, only moisture content, pH, free acidity, electrical conductivity, ash content, and 5-HMF were included in this study. The reason for excluding diastase activity is that it is mostly used for freshness indicators and the samples in this study were freshly extracted; thus, this analysis is not required (Hassan et al., 2023). Besides, sugar analysis is mainly for detecting honey adulteration (Fakhlaei et al. 2020). The honey used in this study is genuine, as the beekeepers have a good manufacturing practice (GMP) certificate.

The Malaysian stingless bee honey samples belonged to the *H. itama* bee species and were collected from three different meliponiculture sites located in the northern region of Peninsular Malaysia (Pulau Pinang, Kedah, and Perak). In this study, storage conditions play a vital role in the changes in the physicochemical characteristics, 5-HMF content, antioxidant properties, and multi-element content in Malaysian stingless bee honey. The different storage conditions are defined by the storage periods (T) and storage temperatures upon analysis. Three different storage conditions of Malaysian stingless bee honey are involved in this study, and they are fresh samples (T= 0), 4 °C (T= 6 months), and 30 °C (T= 6 months). The reason for choosing these storage temperatures (4 and 30 °C) is these are common storage conditions for honey in stores and households where 4 °C represents refrigerator temperature and 30 °C represents room temperature.

First and foremost, the designated physicochemical characteristics, antioxidant properties, quantitative analysis of 5-HMF content, and multi-elements in Malaysian stingless bee honey (T=0) were investigated. Quantitative analysis of 5-HMF and multi-elements were carried out using high-performance liquid chromatography (HPLC) and inductively coupled plasma-optical emission spectrometry (ICP-OES), respectively. An isocratic elution program was used for the quantitative analysis of 5-HMF content. Subsequently, a liquid-liquid extraction (LLE) technique was used for the extraction of volatile organic compounds in Malaysian stingless bee honey and chromatographic fingerprints were generated using GC-MS. With the data of the mentioned analyses at fresh state, the discrimination study was carried out and putative chemical markers were determined using a chemometrics application.

Then, after six months, similar analyses (except for volatile organic compounds) were conducted for the Malaysian stingless bee honey stored at 4 °C and 30 °C. Afterwards, the influence of storage conditions on physicochemical characteristics, antioxidant properties, 5-HMF content, and multi-element in Malaysian stingless bee honey was visualised with the obtained data. Then, the chemometrics approach was used to identify the most significant variables in discriminating between fresh and stored honey. Antioxidant and anti-hypertensive properties of the samples can hardly be justified based on total phenolic and flavonoid content; thus, it was confirmed by reducing power, free radical scavenging activity, and ACE inhibitory assay of selective samples from respective geographical origins.

The limitation of the study includes the GC-MS operational fault, which limits the analysis of volatile organic compounds after the storage period of six months. As a result, the influence of storage conditions on volatile organic compounds cannot be included in this study. In conjunction with this limitation, the identification of putative chemical markers (in terms of volatile organic compounds) to distinguish fresh from stored samples (non-fresh) is unable to be carried out.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of chapter

This literature review segment is about critically analysing and synthesising previous scholarly work pertinent to the study issue. The literature review of this thesis enclosed the fundamental theory of the investigated analyses (i.e., physicochemical characteristics, 5-HMF content, total phenolic and flavonoid contents, multi-element content, volatile organic compounds, antioxidant properties and anti-hypertensive properties) with machine learning (i.e., chemometrics) and the previously reported studies on stingless bee honey to provide better visualisation of the main ideas, concepts, discoveries, and gaps that this study seeks to fill are highlighted in this thorough assessment of the state of knowledge.

2.2 Background of Malaysian stingless bees and their products

The bees responsible for the production of Malaysian stingless bee honey are named kelulut, while its honey is known as kelulut honey (Yaacob et al., 2018). Generally, kelulut is active in the collection of nectar most of the time except during rainy, gloomy, and cold weather (Maringgal et al., 2019). Besides that, kelulut is highly gregarious, with one queen living together with thousands of nurse bees (Maringgal et al., 2019). So far, 32 kelulut species have been reported in Malaysia, such as *Heterotrigona bakeri* (*H. bakeri*), *H. itama*, *Tetrigona apicallis* (*T. apicallis*), *Geniotrigona thoracica* (*G. thoracica*), *Lepidotrigona terminate* (*L. terminate*), *Trigona binghami* (*T. binghami*), *Homotrigona alicae* (*H. alicae*), etc. (Biluca et al., 2016; Ng et al., 2021). Kelulut honey is multiflora honey that can usually be obtained from hollow trees. Recently, there has been growing interest among the farmers to develop kelulut farm with two common kelulut species (*H. itama* and *G. thoracica*) due to the high demand among Malaysians and high selling price compared to other honeybee honey (Sharin et al., 2021).

Volatile and non-volatile (amino acids, organic acids, vitamins, phenolic acids, and flavonoids) compounds in Malaysian stingless bee honey made it gain popularity among researchers and scientists. This is because the presence of these compounds in Malaysian stingless bee honey is the main reason for honey's biological, medicinal, and therapeutic properties (Figure 2.1) (Table 2.1). Apart from being consumed in its raw form, stingless bee honey is widely used in the food and cosmetic industries due to its natural sweetener and moisturizing properties (Figure 2.2).

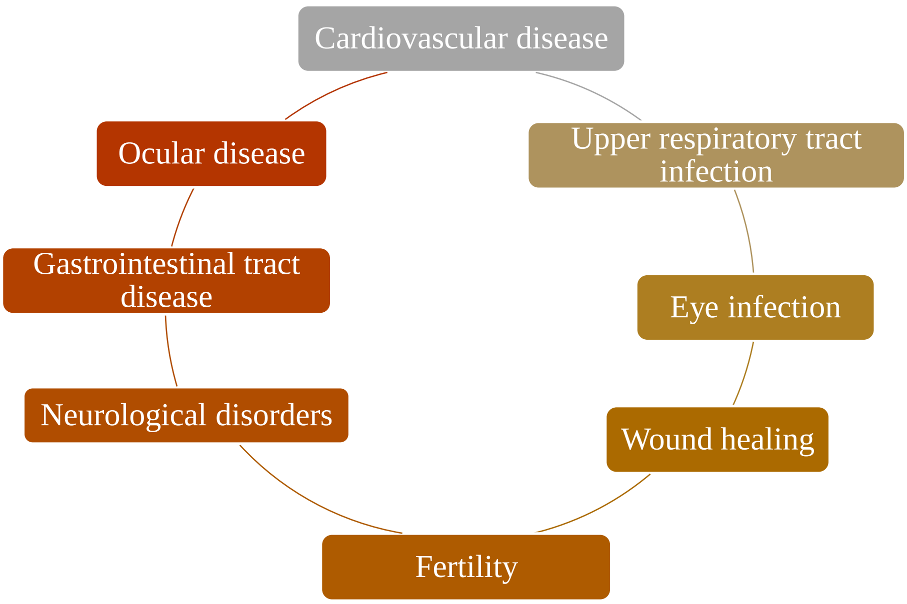

Figure 2.1: Medicinal benefits of Malaysian stingless bee honey (Amin et al., 2018; Jalil et al., 2017; Mustafa et al., 2020; Ramli et al., 2019; Rao et al., 2016)

Table 2.1: Biological properties of Malaysian stingless bee honey

Biological property	Group	Specific/potential compound	Reference
Antioxidant	Flavonoid	Flavanols: Kaempferol, quercetin, rutin, and galangin Flavones: Genkwanin, luteolin, apigenin, tricetin, and chrysin Flavanones: Pinocembrin and pinostrobin	Amin et al. (2018) and Tuksitha et al. (2018)
	Enzyme	Glucose oxidase and catalase	
	Phenolic acid	Gallic acid, syringic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, and ferulic acids	
Antidiabetic	Flavanoid	Flavanols: Quercetin Flavone: Chrysin	Ali et al. (2020) and Vinayagam & Xu (2015)
Antitumor	Phenolic acid	Gallic acid, caffeic acid, and *p*-coumaric acid	Ahmad et al. (2009) and Zulpa et al. (2021)
	Flavonoid	Flavanol: Rutin Flavones: Chrysin and luteolin	
Antiallergic	Flavonoid	Flavanol: Rutin	Aw Yong et al. (2021) and Leong et al. (2021)
Antiproliferative	Phenolic acid	Caffeic acid and gallic acid	Okafor et al. (2021) and Omar et al. (2016)
	Flavonoid	Flavanol: Rutin, kaempferol, and quercetin Flavone: Luteolin	
Anti-inflammatory	Phenolic acid	Gallic acid, caffeic acid, and catechin	Mustafa et al. (2020) and Ooi et al. (2021)
	Flavonoid	Flavanol: Rutin Flavone: Apigenin	

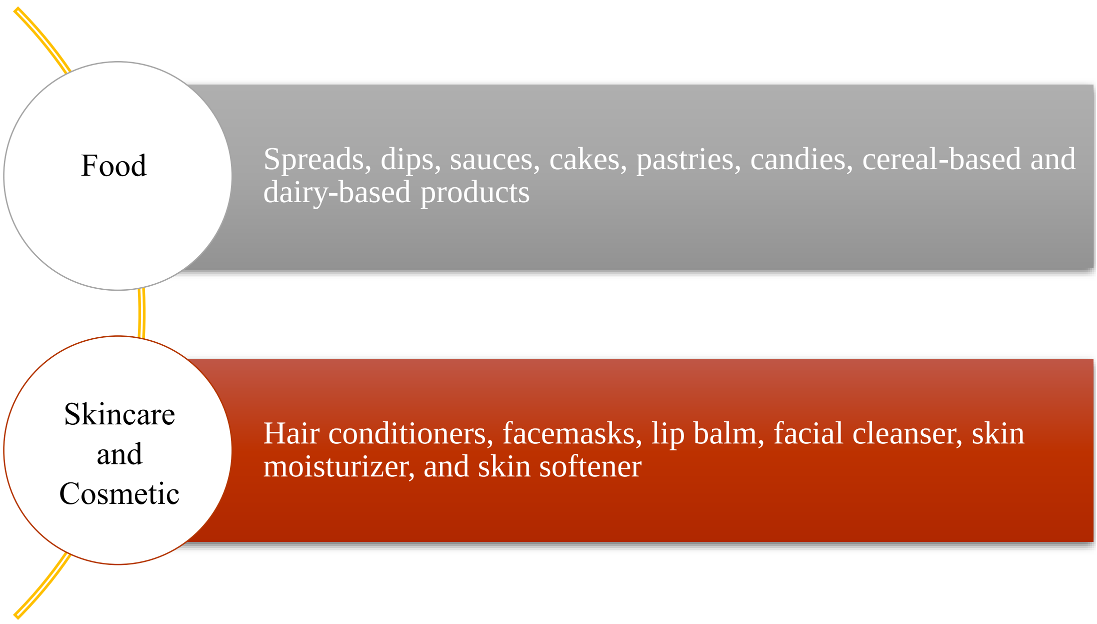

Figure 2.2: The application of Malaysian stingless bee honey in various products (Burlando & Cornara, 2013; Castro-Muñoz et al., 2022)

Although Malaysian stingless bee honey has numerous chemical constituents that offer various health benefits, it must be consumed in the right proportion. According to the American Heart Association, honey should be treated like other added sugars because it is mainly composed of carbohydrates; thus, consuming honey without proper measurement may lead to a high daily calorie intake. The association recommends a maximum honey intake of not more than nine teaspoons for men and six teaspoons for women and children (Johnson et al., 2009). On the other hand, the contents of minerals in stingless bee honey also play a vital role in determining the maximum daily intake (Winiarska-Mieczan et al., 2021). Therefore, the concentration of minerals in stingless bee honey must be identified in order to calculate the intake of these elements via honey. This is because high (more than the required level) consumption of minerals will lead to adverse health issues, including chronic kidney disease (Nkansah et al., 2018).

Other than honey, some other notable products of the Malaysian stingless bee include stingless bee bread, propolis, royal jelly, and beeswax. Stingless bee bread is made up of pollen of blossoms and salivary enzymes of stingless bees, and then the mixture undergoes lactic acid fermentation to form bee bread (Mohammad et al., 2020). Generally, stingless bee bread is more expensive than stingless bee honey because it has less production quantity and has significant antioxidant and therapeutic properties (Mohammad et al., 2020). The price of stingless bee bread can go up to RM 400 per kilogram. The resinous hive product is propolis and it is made up of salivary enzymes and wax of stingless bees, and resins from plants (Farnesi et al., 2009). If the propolis contains minerals and soil, it is known as geopropolis (Lavinas et al., 2019). Humans consume propolis for its biological properties, but it is used as a sealer of honeycombs by stingless bees to prevent air and foreign insects from entering beehives (Lavinas et al., 2019). Beeswax is produced naturally by stingless bees from their specialised wax gland located on the ventral side of the stingless bee's abdomen (Bogdanov, 2004). Last but not least, royal jelly is the secretion of stingless bees from their hypopharynx glands (Hu et al., 2019). The quantity of royal jelly is usually less because larvae and nurse bees consume it as a source of nutrients and energy (Ahmad et al., 2020; Khazaei et al., 2018).

2.3 Physicochemical characteristics

Generally, food properties can be divided into four main classes and they are health, sensory, kinetic, and physicochemical properties (Yousseef et al., 2016). The physicochemical properties are usually used as quality assessment parameters in determining the quality of food products. The physiochemical characteristics commonly and globally assessed on honey are moisture content, ash content, pH, free acidity, electrical conductivity, total dissolved solids, and enzyme activity (invertase and diastase) (Mohammed, 2022).

2.3.1 Moisture content

Moisture content analysis is a type of assay to determine how much water content is present in a product. The analysis of moisture content is vital in food industries, such as honey, because the water particles in food play a leading role in determining the quality of food and microbial stability; this can be explained in terms of the concentration of fermentative bacteria or fungus (Kunová et al., 2015). When the moisture content of honey is high, it triggers excessive growth of osmotolerant yeast (Yegge et al., 2021). The excessive cultivation of osmotolerant yeast will cause spoilage of honey via the fermentation process, as the rate of fermentation is higher (Mesele, 2020). The fermented honey has degraded qualities, such as high acidity and unpleasant appearance; consequently, the durability and shelf-life of honey will be shortened (Ramly et al., 2021).

Several different approaches have been reported in previous studies on determining the moisture content of honey. In the early 90s, the vacuum drying method was used, which involved a vacuum oven and a high vacuum pump (Sancho et al., 1991). With the development of science and technology, Karl Fischer titration and infrared spectroscopy were introduced (Almeida-Muradian et al., 2014; Isengard et al., 2001). However, the titration method is time-consuming and the availability and accessibility of infrared spectroscopy are limited. Thus, a digital refractometer has been introduced and is widely used for the rapid determination of moisture content in honey (Dan et al., 2018).

Three major factors influence the moisture content of honey and they are honey maturity, environmental and climatic conditions, and honey storage conditions (Figure 2.3) (Omar et al., 2019; Shamsudin et al., 2019a). First and foremost, honey in beehives that has been fully matured before being extracted by beekeepers usually has lower moisture content than partially matured honey (known as unripe honey) (Guo et al., 2020). On the other hand, high humidity or rainfalls in the region where honeycombs are located will cause the honey to have high moisture content due to the water particles from the atmosphere and rain being absorbed by honey (Hassan et al., 2021).

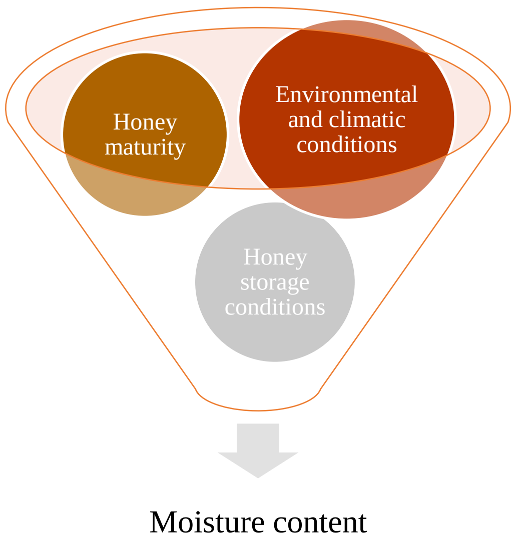

Figure 2.3: Factors that influence the moisture content of honey

The weather or season during honey harvesting also plays a crucial part in determining the moisture content of honey (Al-Farsi et al., 2018). Harvesting honey from honeycomb during summer or hot days is preferred as the honey dehydrates much faster. Moreover, a longer storage period triggers the moisture content level in honey to rise as a consequence of the hygroscopic properties of honey (Julika et al., 2022). As honey is hygroscopic, it has a greater tendency to absorb water particles from the humid atmosphere. The storage temperatures during a long storage period have been reported to have an impact on the moisture content of honey (Kedzierska-Matysek et al., 2016). For example, commercialized Malaysian honey (thermally treated) was reported with low moisture content at a storage temperature below 4 °C compared to commercialized Malaysian honey stored at 25 °C. This can be explained by the weak ability of air to hold water particles at low temperatures; thus, the chemical bond strength of air with water molecules is weakened (Fuad et al., 2018).

Given that various aspects contribute to the changes in the moisture content of honey, an advised range for the moisture content of stingless bee honey has been established locally and internationally in order to preserve the quality and durability of honey. According to Codex Alimentarius Standard and EU Honey Directive, the maximum allowed percentage for moisture content of general honey (including stingless bee honey) is 20% (Bogdanov & Martin, 2002; EU Council, 2002). However, the climate and weather in Southeast Asian countries like Malaysia are different; thus Department of Standards Malaysia has set the moisture content of raw Malaysian stingless bee honey to be up to 35%, while the moisture content of processed Malaysian stingless bee honey to not be more than 22% (Department of Standards Malaysia, 2017).

Comprehensively, the impact of stingless bee honey storage conditions from different regions on moisture content was studied globally to visualise the trend of moisture content of stingless bee honey (Table 2.2). However, all the reported studies do not share a similar moisture content trend under respective storage conditions. Besides, although there is a study on the impact of storage conditions on the moisture content of Malaysian stingless bee honey from *H. itama* species, it involved a single sample only and was not widely investigated (Julika et al., 2022). Thus, a study on the impact of storage conditions on the moisture content of Malaysian stingless bee honey solely from *H. itama* species is vital.

Table 2.2: Trend of moisture content of various stingless bee honey at different storage conditions

Geographical origin	Stingless bee species	Moisture content (%) (T=0)	Trend of moisture content after specific storage period			Comparison	Storage period	Reference
			A	B	C			
Malaysia	*G. thoracica*	33.00	Increased	Increased	Not studied	A < B	90 days	Julika et al. (2022)
	H. itama	30.8	Decreased	Increased	Not studied	A < B		
Brazil (Salvador)	*M. scutellaris*	26.00	Decreased	Decreased	Not studied	B < A	150 days	Silva et al. (2021c)
Thailand (Pattani)	*T. larviceps*	25.40	Increased	Increased	Not studied	A < B	45 days	Meechai et al. (2021)
Malaysia (Selangor)	*G. thoracica*	26.03	Not studied	Decreased	Decreased	C < B	7 days	Mohamad et al. (2021)
Malaysia (Terengganu)	-	18.59 – 29.61	Decreased	Decreased	Not studied	A < B	300 days	Fuad et al. (2018)
Thailand (Chiang Mai)	*T. laeviceps-pagdeni*	24.70	Increased	Increased	Increased	A < C < B	12 months	Chuttong et al. (2016)

T: storage time; A: stored at ≤ 4 °C; B: stored at room temperature (25 – 30 °C); C: stored at ≥ 35 °C; *G. thoracica*: *Geniotrigona thoracica*; *H. itama*: *Heteotrigona itama*; *M. scutellaris*: *Melipona scutellaris*; *T. larviceps*: *Tetragonula laeviceps*; *T. laeviceps-pagdeni*: *Tetragonula laeviceps-pagdeni*

2.3.2 Ash content

In food studies, ash content analysis is extensively applied to identify the total amount of inorganic minerals within a food, especially in honey, as it is a significant criterion for honey quality (Harris & Marshall, 2017; Majewska et al., 2019). The high temperature during the analysis will burn off the organic content and leave only the inorganic minerals (Nesa et al., 2021; Pyar & Peh, 2018). The amount of residual (inorganic minerals) from the analysis is swayed by the floral and geographical origins (Albu et al., 2021; Imtara et al., 2018; Sousa et al., 2016). When the nectar-bearing plant varies, the ash content varies, as different plants possess different compositions of minerals (Shamsudin et al., 2019a). Thus, nectar and honeydew honey can be easily distinguishable by assessing ash content data (Majewska et al., 2019). On the other hand, as the geographical origin of the honey source differs, the ash content also varies due to the soil type variability (Mouhoubi-Tafinine et al., 2018). Besides that, honey extraction, beekeeping techniques, and substance accumulated by honeybees during foraging on flora also influence the ash content (Kek et al., 2017; Nigussie et al., 2012).

Usually, the ash content parameter is associated with other parameters of honey. Several studies have shown that the ash content of honey has a correlation with honey physicochemical parameters, such as electrical conductivity and colour. Al et al. (2009) and Shafiee et al. (2014) have reported that light-coloured honey has lower ash content, whereas dark-coloured honey has higher ash content. Albu et al. (2021), Kropf et al. (2008), and Majewska et al. (2019) have demonstrated an intense positive relationship between electrical conductivity and ash content; the higher the honey ash content, the higher the electrical conductivity of honey.

Similar to moisture content, the advised range for ash content of stingless bee honey is different locally and globally. The disparity in the recommended range is mainly due to the variability of soil and floral origin. According to Codex Alimentarius Standard and EU Honey Directive, the ash content of general honey (including stingless bee honey) must be ≤ 0.6 g/100 g (Bogdanov & Martin, 2002; EU Council, 2002). Conversely, the Department of Standards Malaysia has proposed that the ash content of raw and processed Malaysian stingless bee honey must be ≤ 1 g/100 g (Department of Standards Malaysia, 2017). Although there is a difference in the recommended limit by international and Malaysian standards, an ashing method is used locally and internationally for the determination of ash content.

Heretofore, only Silva et al. (2021c) has reported the effect of storage conditions of stingless bee (*Melipona scutellaris* (*M. scutellaris*)) honey from Brazil on ash content at two different storage temperatures (5 °C and 30 °C) over stipulated period (Figure 2.4), and the outcome was no significant changes. Besides, the studies on Malaysian stingless bee honey only explored the ash content of honey in the fresh state (Table 1.3).

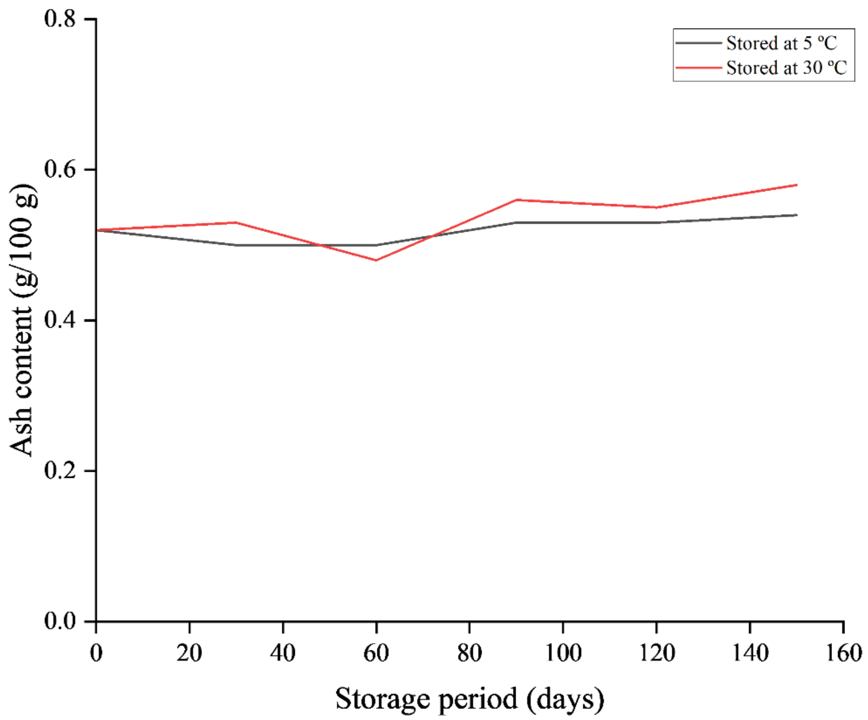

Figure 2.4 Trend of ash content of stingless bee (*M. scutellaris*) honey from Brazil Silva et al. (2021c)